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Plasma membrane reorganization induced by tumor promoters in an epithelial cell line

(fluorescence recovery after photobleaching/phorbol esters/cell shape/cell surface/extracellular matrices)

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ABSTRACT **The effects of phorbol ester tumor promoters on the lateral diffusion in plasma membrane lipid environments were examined by the technique of fluorescence recovery after photobleaching. To this end, the probe collarein, a fluorescent lipid analog that has the property of exclusive localization in the plasma membrane, was synthesized. Measured decreases in three parameters [percentage of fluorescence bleached (30%), percentage of recovery (52%), and half-time for recovery (52%)] connoted the appearance of an immobile fraction upon exposure to tumor promoters. These data are consistent with lipid reorganization in response to a reorganization of the intra- and perimembranous macromolecular scaffolding upon the interaction of cells with tumor promoters. The idea of induced reorganization is supported by experiments in which cell shape change, brought about by either exposure to cytochalasin B or growth on matrices of collagen, fibronectin, or laminin, resulted in values in the fluorescence recovery after photobleaching technique similar to those with active phorbol esters.**

Membranes are dynamic structures in which proteins, glycoproteins, and lipids move *inter alia*. Rates and types of molecular movements are dependent on a combination of molecular class and organization (both local and global) of the particular membrane under study (1, 2).

A considerable amount of evidence suggests that the plasma membrane is intimately involved with the primary events of malignant transformation (3). As a model for studying the transformation process, we chose to examine the effects of the tumor-promoting phorbol esters on an epithelial cell line in culture. Although treatment with tumor promoters alone is not sufficient to induce permanent, irreversible transformation, many of its induced properties are analogous to those caused by transforming agents such as RNA tumor viruses (4–6). In Madin–Darby canine kidney (MDCK) cells, tumor promoters have been shown to effect a malignant-like state in culture (7, 8).

Manifestations of the tumor-promoting activities of phorbol esters have been observed in a broad range of morphologic, biochemical, and biophysical investigations (4–6). Most organelles, many enzymatic pathways, and the cellular surface have been implicated as targets for these agents. Alterations in the following plasma membrane-associated phenomena have been correlated with phorbol ester-induced transformation: cell shape (9), enzymatic activity [e.g., protein kinase C (10, 11) and proteins involved in prostaglandin synthesis (12)], hexose transport (13), growth factor receptor affinity (14), phospholipid and protein synthesis (15, 16), membrane rigidity (17), cellular permeability (7), electrical resistance (7), and metabolic cooperativity (18).

The major objective of this study has been to study the effects of tumor promoters on the lateral diffusion of lipophilic molecules in the plasma membrane of MDCK cells; achievement of a more global view of membrane-associated events was desired in order to ascertain whether modifications in the lipid phase of the membrane could account for some of the phenomena listed above.

MATERIALS AND METHODS

Cultures. MDCK cells were grown to confluence on glass coverslips (≈10 × 22 mm) in 35-mm plastic Petri dishes (Falcon 3001) at 37°C. The medium used for all experiments unless otherwise indicated was minimal essential medium (ME medium) (GIBCO) containing 20 mM Hepes (Sigma) buffer and 10% fetal calf serum at pH 7.4 and 310 mosM.

For experiments involving components of the extracellular matrix, coverslips were coated with collagen by the method of Klebe *et al.* (19) or with fibronectin or laminin by drying ME medium solutions of either glycoprotein on coverslips.

For phorbol ester (Consolidated Midland, Brewster, NY) experiments, serial dilutions were made from stock solutions of 1 mg of phorbol ester per ml of methanol into ME medium; ME medium containing 0.005% methanol served as the control. Phorbol 12-tetradecanoate 13-acetate (TPA), phorbol 12,13-didecanoate (PDD), and 4α-phorbol 12,13-didecanoate (4α-PDD) were the phorbol esters used in this study. Final concentrations of phorbol esters ranged up to 0.812 mM—i.e., 50 ng/ml for TPA.

Cells were exposed to tumor promoters at 50 ng/ml for 2 hr at 37°C (see Fig. 3), to cytoskeletal inhibitors [either cytochalasin B (Sigma) at 0.50 μg/ml (1.04 μM) or colchicine (Sigma) at 39.9 ng/ml (0.1 μM)] for 1 hr at 37°C, or to these solutions sequentially.

Dye. Collarein, a water-soluble fluorescent phospholipid, was synthesized by stirring lissamine rhodamine-B sulfonyl chloride (Molecular Probes, Junction City, OR) and cardiolipin (P-L Biochemicals or Sigma) in a 2:1 ratio in dry pyridine for 24 hr at room temperature. After removal of the solvent, the product was spotted on silica gel plates, which were developed in chloroform/methanol/water, 65:25:4 (vol/vol). The single spot with an R_f value of 0.70 was scraped and showed spectral properties consistent with the structure drawn in Fig. 1.

Subsequent to final drug exposure(s), MDCK cells were kept at room temperature for all succeeding procedures. They were washed twice with serum-free ME medium; this was followed by a 17-min incubation with collarein in ME medium (OD = 2.83; path length = 1.0 cm). The cells were then washed six times with ME medium containing 10% fetal calf serum; coverslips were removed from Petri dishes,

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Abbreviations: MDCK, Madin–Darby canine kidney; TPA, phorbol 12-tetradecanoate 13-acetate; PDD, phorbol 12,13-didecanoate; 4α-PDD, 4α-phorbol 12,13-didecanoate.

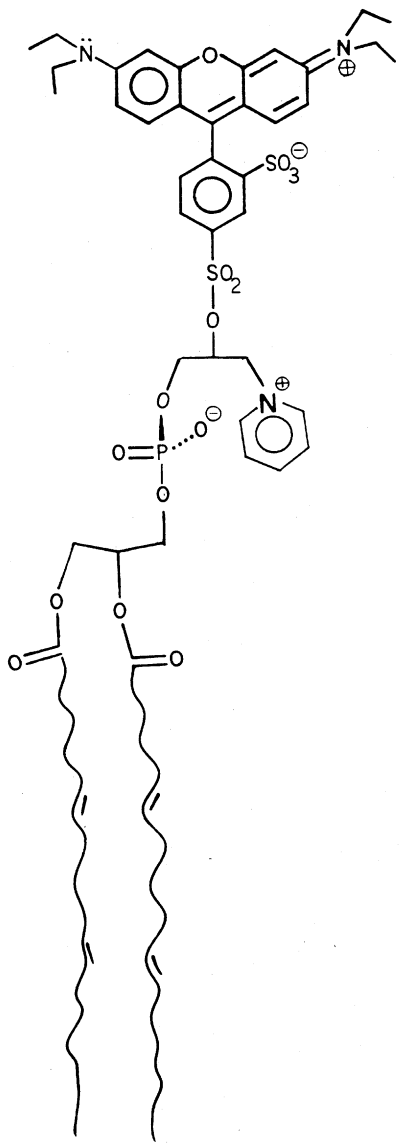

FIG. 1. Collarein.

placed on the microscope stage, and examined for cellular integrity and fluorescence.

The fluorescence lifetime of collarein was fit by a single exponential component (within the current limits of resolution of the instrument) in both control and TPA-treated cells by the method we have described (20); this is consistent with the image observed under the fluorescence microscope of fluorescence confined exclusively to the plasma membrane.

Photobleaching Apparatus. The photobleaching apparatus and method of analysis are similar to those reported previously (21, 22). Briefly 1 W or less of 514-nm light from a 4-W argon ion laser (Lexel) was threaded through a beam splitter/attenuator and thence a Zeiss microscope (×60, 0.85 n.a. oil-immersion objective); the gaussian beam of *ca.* 1.6-μm radius was focused on a plasma membrane by epi-illumination. Fluorescence from the focused spot was excited by the laser beam, which had been attenuated by at least 100-fold to establish a base-line value. The attenuation was removed for 50 msec, resulting in the irreversible destruction of a fraction of the fluorescence in the focused area. The recovery of fluorescence in this area was then monitored, and the three parameters characteristic of this class of experiments were measured: the percentage of bleach, the percentage of recovery, and the half-time for recovery to a steady-state level. The same spot was bleached multiple times subsequent to this recovery, and the three parameters were measured for each bleach pulse or hit.

RESULTS

Our initial photobleaching experiments indicated significant alterations in the lateral diffusion of collarein in cells treated with the phorbol esters TPA and PDD as compared with untreated cells. Relative to the control cells, decreases in the following parameters were measured (for the first hit per spot) with either agent: percentage of bleach, percentage of recovery, and half-time for recovery (Table 1). For the treated cells, as the hit number per spot was increased (subsequent to recovery of fluorescence to a steady-state level), the percentage of bleach decreased while the percentage of recovery increased (Fig. 2 *Right*). The latter usually approached 100% by the fourth hit; thus, the mobile fraction, the percent of collarein with 100% mobility remaining after multiple bleaches, could be determined. Both parameters remained relatively constant for the control cells (Fig. 2 *Left*). Lower concentrations of TPA resulted in intermediate values. Such behavior was in marked contrast to that exhibited by cells after exposure to the inactive congener 4α-PDD, by control cells, or by cells treated with colchicine (see below).

Levels of cellular differentiation and growth have been shown to correlate with cell shape (23–25). The latter can be modulated by the exogenous extracellular matrix components collagen, fibronectin, and laminin, which in certain

Table 1. Photobleaching data (first hit per spot)

Experiment*	% bleach	% recovery	$t_{1/2}$, sec	$D \times 10^{-9}$, cm-cm/sec
1. Control (43)	76.3 ± 3.40	98.5 ± 1.10	4.80 ± 1.20	2.88 ± 0.72
2. TPA (33)	53.2 ± 16.1	47.2 ± 4.40	2.30 ± 0.60	5.08 ± 0.33
3. PPD (18)	55.6 ± 8.40	53.2 ± 13.7	2.80 ± 1.00	4.24 ± 1.51
4. Colchicine (21)	82.3 ± 5.00	100.0 ± 0.00	5.40 ± 0.70	2.76 ± 0.36
5. Colchicine, TPA (13)	48.3 ± 10.2	28.2 ± 8.70	1.73 ± 0.33	6.60 ± 1.26
6. TPA, colchicine (20)	48.6 ± 13.7	40.9 ± 20.7†	1.90 ± 0.50	6.04 ± 2.63
7. Cytochalasin B (7)	65.3 ± 13.1	64.4 ± 23.7	4.21 ± 4.90‡	2.96 ± 3.45
8. Cytochalasin B, TPA (10)	49.9 ± 15.5	35.3 ± 14.7	1.70 ± 0.50	6.04 ± 1.78
9. TPA, cytochalasin B (10)	53.1 ± 10.6	29.0 ± 9.30	1.70 ± 0.60	6.88 ± 2.43
10. Collagen (13)	45.9 ± 6.60	52.8 ± 19.4	2.50 ± 1.20§	4.76 ± 2.28
11. Fibronectin (15)	47.5 ± 1.50	40.9 ± 4.10	2.30 ± 1.00	4.96 ± 0.22
12. Laminin (22)	45.3 ± 11.2	56.5 ± 3.60	4.10 ± 1.10	2.76 ± 0.74

The data (means ± SD) reported in the respective columns give the cell treatment, the percentage of fluorescence bleached by the first of a series of bleach pulses, the percentage of fluorescence recovered, half-time ($t_{1/2}$) for recovery, and the self-diffusion coefficient derived therefrom. All values are within 95% confidence limit except those indicated † (90%) and ‡ (50%).

*Numbers in parentheses are the number of hits used for data in this table. (These numbers correspond to the number of individual cells examined, the number of distinctly different spots on a particular cell, or a combination of both.)

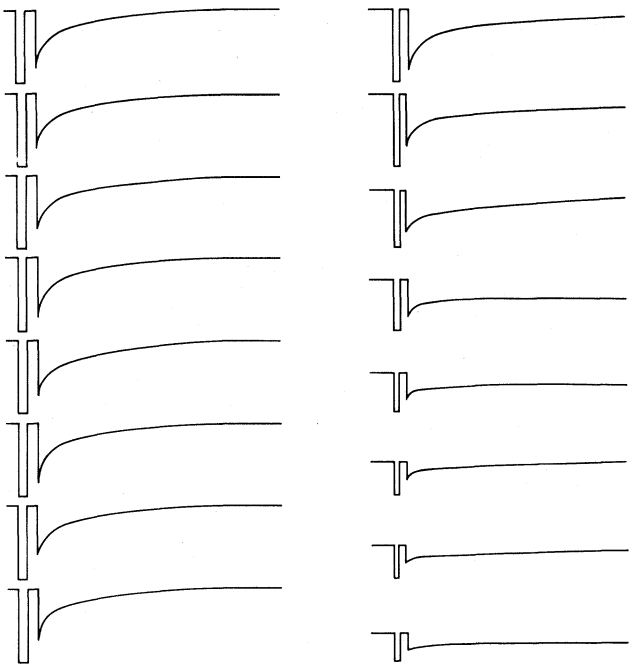

FIG. 2. Fluorescence recovery after photobleaching trace. (*Left*) Eight successive hits and recoveries on the same spot of a control cell. (*Right*) Eight successive hits and recoveries on the same spot of a TPA-treated cell.

systems are able to make cells flatten or fold or to cause in general some perturbation in cellular shape, thereby implying a change in the intramembranous and often submembranous molecular organization. The cytoplasmic cortical elements also can be perturbed directly by the cytoskeletal-disrupting agents cytochalasin B and colchicine; treatment with either may alter the cellular morphology.

To determine if the results obtained with the active phorbol esters were due to a change in cell shape (Fig. 3), we induced morphologic alterations of the MDCK cells with both extracellular and intracellular perturbing agents—i.e., by growth on substrata composed of the extracellular matrix components collagen, fibronectin, or laminin and by incubation with the microfilament inhibitor cytochalasin B, respectively. Additionally, we exposed cells to the microtubule inhibitor colchicine; however, with this compound, no obvious cell shape change was detected.

Growth of cells on collagen or fibronectin reduced the percentage of recovery and half-time for recovery to values obtained with the active phorbol esters, whereas incubation with cytochalasin B and growth on laminin reduced these parameters to values intermediate between those of the control and active phorbol ester-treated cells. Growth on the three substrata resulted in an even greater decrease in the percentage of bleach.

Incubation with a combination of either cytoskeletal inhibitor and TPA treatment, irrespective of order, showed the behavioral characteristics induced by TPA or PDD at 50 ng/ml.

DISCUSSION

We have used the effects of the tumor-promoting phorbol esters TPA and PDD on MDCK cells as a means for understanding the mechanism of chemical transformation in culture. In the belief that the motions of lipophilic molecules in the plasma membrane bilayer might be transformation sensitive, we have designed and synthesized a fluorescent probe molecule that localizes exclusively in the plasma membrane (20).

In cells treated with active phorbol esters, the probe reports the induction of a second membrane environment that is characterized by a significantly lower mobility. Similar changes can be mimicked by altering the cell shape either intracellularly by perturbation of the microfilamentous orga-

FIG. 3. MDCK cells as visualized under Nomarski optics. (*Left*) Control (0.005% methanol). (*Right*) After TPA treatment. (Bar = 1 μm.)

nization with cytochalasin B or extracellularly by altering the substratum on which the cells adhere and grow. These data are consistent with the lateral diffusivity of some fraction of the lipophilic fluorophore collarein being strongly influenced by the organization of the larger and relatively immobile proteinaceous integral and peripheral structure of the plasma membrane. More specifically, in the untreated cell, the membrane-associated proteins may assume a fairly homogeneous distribution; upon treatment with an active tumor promoter, an intramembranous condensation of some of the molecular elements may occur, rendering them relatively immobile. The lipids then may respond to this "reorganized" relatively immobile structure by forming domains characterized by degrees of mobility. Some collarein molecules may get "caught" in condensed regions; this could lead to an immobility on the photobleaching time scale. Others could be excluded from these regions, and their diffusion coefficients would increase because of the increased lipid-to-protein ratio of the environment they are then probing. Thus, in the process of intramembranous "condensation," a more heterogeneous intramembranous scaffolding may appear, resulting in a change from a relatively homogeneous distribution of lipid molecules to a heterogeneity of environments. This reorganization would be reflected in the mobility measurements reported here as a decreased fraction of recovery, an increased diffusion coefficient for molecules still free to diffuse because of the relatively increased local lipid/protein ratio, and perhaps a decreased fraction of molecules accessible to bleaching by the polarized laser light.

This interpretation is consistent with previous work that has examined membrane changes associated with treatment of tumor-promoting phorbol esters. Jacobson *et al.* (26) observed no differential effects between the active and inactive phorbol esters in dipalmitoyl phosphatidylcholine model membranes on the main transition temperature, fluorescence anisotropy, cationic permeability, electrophoretic mobility, or conductance of planar bilayers. In contrast, when whole cells have been examined subsequent to TPA treatment, a picture of membrane susceptibility emerges. For example, Shoyab *et al.* (14) have reported a decrease in the affinity of the epidermal growth factor receptor for the factor subsequent to TPA treatment. The fluorescence anisotropy of the fluorophore diphenylhexatriene appears to correlate with TPA sensitivity of susceptible and resistant murine erythroleukemic cell clones (17). Thus, TPA appears to affect membrane lipid properties of cells but not lipids in nonprotein-containing bilayers.

Control of molecular diffusion in biologic membranes by the matrix of macromolecules associated with plasma membranes has been demonstrated by the measured increases in diffusion rates of both lipids and proteins in spectrin-deficient spherocytic mouse erythrocytes compared with normal cells (27), by changes in both macro- and microscopic states of intramembranous particles upon collapse of erythrocyte ghost cytoskeletal elements (28), and by a reduction in protein mobility in the absence of any effect on the mobility of a lipid probe in L-6 myoblasts subsequent to treatment with cytochalasin B (29). Additional examples of altered mobilities upon release of constraints also have been reported (30, 31).

Changes in dynamic interactions of molecules embedded in the plasma membrane may be an indication of or a means for major physiologic alterations at the molecular level (32, 33). Examples include changes in lateral diffusion rates of fluorescent lipid analogs in mouse egg plasma membranes upon fertilization and first cleavage (34) and in the mobile fraction of viral surface proteins upon activation of viral expression (35).

In conclusion, we believe the primary biophysical membrane effect of TPA is on the protein and protein-associated structure of plasma membranes. Furthermore, the increased diffusion coefficients of the mobile fraction that we observe upon chemical transformation or treatment with inducers of cell-shape change appear to reflect changes in media organization. Thus, collarein, a lipophile, is probing an environment that is responding to a reorganization of the larger, more immobile membrane-associated molecules.

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